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THE FORMATION OF CYTOPLASMIC PARTICLES DURING CELL GROWTH AND DIVISION

By

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The extra-nuclear portion of the cells of higher organisms (the cytoplasm) contains several major particulate elements. In most cells, the mitochondria and the endoplasmic reticulum constitute the most prominent particulates, while green plant cells also contain chloroplasts. Young cells usually do not contain a prominent endoplasmic reticulum, but contain instead a number of small ribonucleoprotein particles (ribosomes). As the cell matures, the extensive membrane system of the endoplasmic reticulum is formed, and the ribosomes are seen to be attached to the membrane network. (Porter, 1962)

An integral part of the formation of new cells during the growth of an organism is the formation of new cytoplasmic particulates. We have initiated an investigation of the manner in which the major particulates of the cytoplasm are formed. The results, although still in an early state, are indicative of a highly unified method for the formation of the major cytoplasmic particulates.

Formation of ribosomes. Ribosomes are spherical particles with diameters of 200–300 Angstroms and molecular weights of 3–4 million. They are composed of approximately equal amounts of protein and ribonucleic acid (RNA). Ribosomes from various organisms are remarkably similar in structure and composition (Webster and Whitman, 1963). The protein portion of ribosomes is built up from a large number of a few kinds of protein sub-units of molecular weight of about 25,000. The RNA portion consists of one or two large RNA molecules. In addition to the ribosomal RNA itself, ribosomes usually have some messenger RNA and some amino acid transfer RNA associated with them.

The question, therefore, is where and how the ribosome is formed. By use of the large, single-cell marine alga, *Acetabularia crenulata*, we have obtained clear evidence that the protein of ribosomes is formed in the cytoplasm, probably by other ribosomes (Webster, *et al.*, 1962). *Acetabularia* cells can easily be enucleated, and we have found that cells lacking a nucleus are just as capable of forming

ribosomal protein as cells which have a nucleus. Further experiments are contemplated to determine with certainty whether ribosomal protein is indeed formed by ribosomes.

Similar experiments have shown that ribosomal RNA is likewise formed in the cytoplasm of cells lacking a nucleus (Sutter, *et al.*, 1961). This cytoplasmic synthesis proceeds for only about ten days following the removal of the nucleus (Webster, *et al.*, 1962), however, and it is not at all certain that ribosomal RNA is normally formed in the cytoplasm. In any event, the formation of an intact ribosome from ribosomal RNA and ribosomal protein sub-units is probably a spontaneous process, because ribosomal protein sub-units have been shown to aggregate together spontaneously under proper conditions to form an intact ribosome in much the same way that an intact tobacco mosaic virus can be formed from the viral RNA and protein sub-units (Fraenkel-Conrat and Williams, 1955).

Formation of endoplasmic reticulum. The endoplasmic reticulum consists of two major structural elements : a network of membranes, and, attached to the membranes, the ribosomes. The formation of the reticulum during the maturation of cells is apparently the result of the net synthesis of the entire network of membranes. The structure of these membranes, and the manner in which they are formed are essentially unknown. In fact, little is known about the reticulum membranes except that they contain a number of different enzymes. The best possibilty for their formation is that they are synthesized by the ribosomes (which are known to be major sites of protein synthesis in the cell). The determination of the manner of synthesis of the membranes, however, must await the determination of their structure and composition.

Formation of mitochondria. The possibility that mitochondria are self-replicating bodies has been entertained for a long time, and this possibility has been strengthened by the reports of a number of investigators that isolated mitochondria are able to incorporate amino acids into proteins under conditions which allow oxidative phosphorylation to proceed. However, recent studies with liver mitochondria produced the puzzling finding that isolation of three different proteins from mitochondria which had incorporated significant amounts of amino acid revealed that none of these proteins contained incorporated amino acid (Roodyn, *et al.*, 1962). This suggested either that only certain proteins are formed by the mitochondria under the experimental conditions, or that the amino acid incorporation which has been measured previously is an artifact and is not the result of mitochondrial protein synthesis. We, therefore, have examined the ability of mitochondria to form the various proteins of which the mitochondria are composed. The isolation of highly-active mitochondria from heart muscle has been developed to a high degree in this laboratory (Green, 1963). Such mitochondria are able to oxidize substrates of the Krebs cycle and to form ATP very efficiently. If mitochondria are able to form proteins, these mitochondria should be capable of such synthesis.

Mitochondria from heart muscle are composed principally of a membrane structure which forms a supporting vehicle for small particles (elementary particles) which perform the oxidation process. The membrane structure is composed of a network of phospholipid and a specific, low molecular weight, structural protein. A major portion of the mitochondrial portein is this structural protein. The elementary particles consist of flavoprotein dehydrogenases and cytochromes *a*, *b*, *c*, and c_1 (Green, 1963).

We have found that these mitochondria readily take up amino acids, and by the criteria used by previous investigators, incorporate the amino acids into

mitochondrial protein. However, when each of the major components of the mitochondrion (structural protein, cytochromes *a, b, c,* c_1, or the entire elementary particle) is isolated, the purification process is accompanied by a complete loss of incorporated amino acid. The amino acid is found in the lipid fraction wh ch is removed during purification. Therefore, it has not been possible to find any evidence that mitochondria are capable of forming their constituent proteins or of replicating themselves. The ease with which mitochondrial components can be reconstituted into more complex functional structures has led to the suggestion (Green, 1963) that mitochondria are formed by a spontaneous aggregation of their component parts. The inability of mitochondria to replicate these parts raises the question of where the parts are formed. At present, there is no information on this question.

Therefore, the formation of the major structures of the cytoplasm appears to be a problem that is now amenable to solution. Some of the misconceptions regarding the manner in which these structures are formed have been removed, and experimental approaches to the problems are available. Within the next several years considerable information should appear regarding these questions.

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GENESIS AND GROWTH OF PSEUDOTUMOURS (MELANOTIC TUMOURS) IN DROSOPHILA

By

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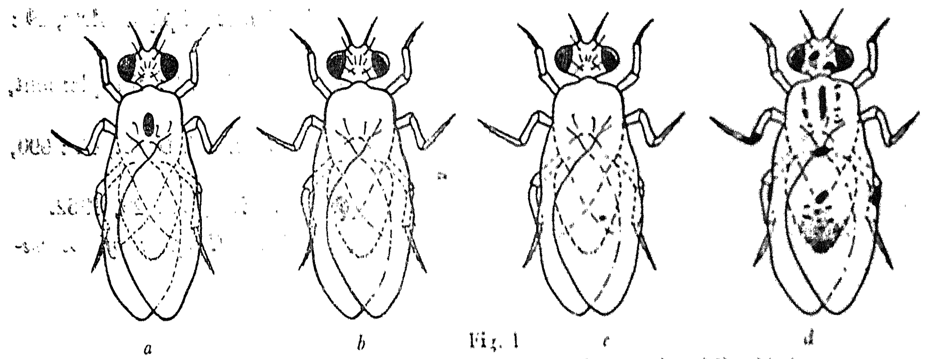
The melanotic tumours of Insects, and especially of Drosophila, are black formations, sometimes caused by external injuries (Castiglioni a. Beati, 1954) and sometimes by internal factors (genes and cytoplasmic units : for a general survey of the problem see : Barigozzi, 1961).

The black masses are attached to viscera, and are sometimes so large as to occupy a considerable part of the abdomen. Even in these conditions, melanotic tumours do not cause any apparent harm to the carrier, except in a few cases. In the majority of the stocks, melanotic tumours are very rare (only exceptionally a stock is completely tumourfree) while in several stocks they can reach spontaneously very high percentages and even 100%. Melanotic tumours can be present in one or more masses. In stock tu A_2 the most general pattern consists in the presence of one or few abdominal masses : in nearly 15% an additional tumour is present located in the middle of the thorax (fig. 1*a*). Very peculiar is the aspect of stock Freckled (a dominant character) where tiny black masses (besides some larger ones located in the abdomen) are scattered throughout the whole body including the head, forming very peculiar sequences along the aorta or other organs (see fig. 1*d*).



a *b* Fig. 1 *c* *d*

a stock tu A_2 ; *b* stock tu P_3 ; *c* almost tumourless stock ; *d* Freckled

The production of these masses poses the problem of their nature and of their growth. Both are discussed in the present paper.

The nature and the formation of the melanotic tumours :

(*a*) The nature of the melanotic mature tumours is easily understood studying their structure. In all stocks investigated so far (especially when tumours are not located in the fatbodies, where the conditions might be different, and with the exclusion of those referred to as "malignant" studied by El Shatoury, 1955*a*,*b*), the tumour contains very few cells embedded in a large amount of a black and breakable substance, which proves to be melanine (Röhrborn, 1962). Hence it is out of question that nearly all melanotic masses, which the author has proposed

to call *pseudotumours*, are not real tumours. Moreover they are a production of the cell metabolism.

(*b*) The formation of an inherited melanotic tumour does not follow a uniform process. We know nonetheless a majority of stocks where the melanine clump is the product of the following series of phenomena :

1. release of cells from the lymph gland
2. congregation of a given type of haemolymph cells (the large ones, see later) and change of their shape, which becomes thinner and flat.
3. production of melanine by the congregated cells, which remain embedded within the melanine (Castiglioni, 1957). The first step occurs as a physiological fact prior to any tumour formation ; the second one is actually the initiation of the tumour and brings to the formation of colourless masses, referred to as *pretumours*. The release of cells from the gland is connected with the structure of the gland itself, which becomes loose and even more of less largely disintegrated during the larval life (Fig. 2).

Although it is not excluded that multiplication of a given cell type may be particularly active under the influence of the hereditary factors which control tumour production, the phenomena described bring to conclude that the formation of a tumour in Drosophila is not predominantly caused by cell multiplication, but by congregation of cells already present, and that the cell type capable of producing melanine is a normal one. These facts mark a clear cut difference with neoplastic growth.

For a better understanding of the findings described above, it is necessary to say a few words about the structure of the lymph gland and of the cellular elements of the haemolymph.

The lymph gland (Castiglioni 1955, 1956, 1957) is a bilateral organ (not completely symmetrical in its two halves) located along both sides of the aorta. Each half consists in a series of solid lobes, the anterior being generally larger than the posterior ones. Within each lobe there are a number of cells, which show different shapes and structures. Their analysis is difficult until they leave the gland : generally, this occurs during the larval life. In many tumorous stocks the gland loses the majority of their cells, thus looking as disintegrated. Disintegration is completed before pupation, with great individual variation. In the tumour free stocks the gland may remain intact. In any case, the gland lacks in the adult.

The cells released from the gland swim freely in the haemocoele. It is possible to count and to classify them. Their number increases during the larval development as shown on Table 1 for the stock melanotic e 144 (Halfer 1961). In the same stock it has been found that the total number of haemolymph cells proved to be related to the tumour incidence, and to change through selection.

Selected lines reaching a very high tumour frequency had per full grown larva (mm. 4·5–5) an average of 3268·8 cells (from the total amount of lymph, collected through dissection), versus an average of 1223·8 in the control and of 1229·3 in a line selected for low tumour frequency. This means that cells multiplication and cell release are genetically controlled.

Regarding the different cell type of the haemolymph, there is no full agreement as far as the classification is concerned.

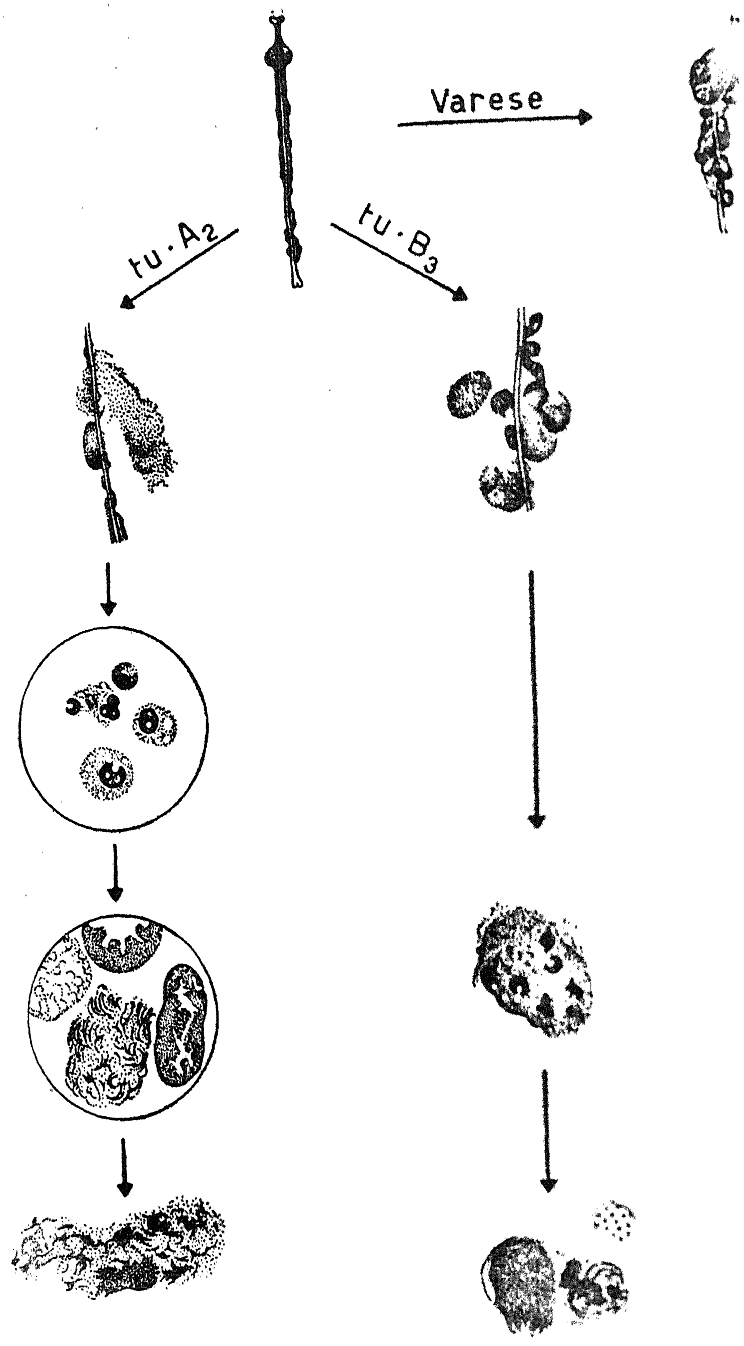


Fig. 2

Scheme of formation of a pseudotumour in stocks tu A_2 and tu B_3 ; lymph gland remains intact in tumorless varese and disintegrates in tu A_2 and tu B_3.

TABLE 1

Total number of haemolymph cells and means of the different cell types per individual of larvae of different length (stock melanotic e 144)

Length	n. of cells	Percentag of cells Small	Mid sized	Large	Crystalloid
mm. 3–3,5	794	18,1	63,9	15,7	2,3
mm. 3,5–3,9	1700	27,3	58,8	10,9	3,5
mm. 4·0–4,4 without tumour	4035	30,7	62,8	3,9	2,6
mm. 4,5–5·0 without tumour	4652	32,3	59,8	4,7	3,2
mm. 4·0–4,4 with tumour	3243	41,2	54,3	3,1	1,4
mm. 4,5–5·0 with tumour	6119	43,6	50,0	4,6	1,8

One scheme is proposed by Rizki (1962), who distinguishes only two main types of cells : the plasmatocytes and the crystal cells. The former ones (constituting the 90–95% of the whole haemocytes) show two variant : the podocytes, characterized by pseudopodes, and the lamellocytes, which are flat and large. The findings are based on observations *in vivo*. The other scheme, proposed by Castiglioni (Castiglioni 1957 ; Barigozzi, Castiglioni and Di Pasquale 1960), based on stained material, is not fundamentally different. It distinguishes the non crystalloid cells in three variants : small cells, mid sized cells, large cells. The last ones (presumably corresponding to the lamellocytes of Rizki) in the stocks studied by our group proved to be the only type capable of producing melanine, after a change of shape : they become very thin, flat and lanceolate.

For this reason the interest has been mainly focussed on the large cells and many countings have been made. It turned out (Table 2) that the tumourous stocks behaving in the way described hitherto, have at least nearly 2% of large cells when the larvae are in the 3d, instar The changes of frequency of the large cells during the larval development is shown on Table, 3 which permits the following remarks :

1. one stock is very precocious in having a detectable frequency of large cells (tu–SoC), and another is very late (tumourfree ltr/ltr).
2. in some stocks there is a decrease of large cells during the larval development, stock tu B_3 shows an increase, and the tumourfree stocks there is constance or irregular changes of the cell number.

The tendency to numeric reduction shown by the large cells is interpreted easily as a result of their clumping to form tumours. Where there are no tumours, no large cells are kept out from swimming freely in the haemolymph.

TABLE 2

Frequency of haemolymph cells in larvae of different stocks of Drosophila melanogaster belonging to different ages within the 3^d instar

Genotype	% of tumours	Small basophilic	% Cell types Mild sized	Large	Crystalloid	n. larvae	n cells
Varese	0·0	23·7	73·5	1·7	1·1	20	7575
S. Maria	2·7	26·0	71·4	1·7	0·9	20	18032
tu–SoC	3·1	24·2	69·6	4·2	2·0	50	24922
b cn vg	5·3	20·1	71·8	4·5	3·6	20	15459
tu–A_2 (2° cr. tu)	85·2	32·3	53·4	12·4	1·9	20	17136
tu–B_3 (2° cr. tu)	66·5	12·6	65·7	19·1	2·6	20	26029
tu–mwh	4·5	12·3	68·0	16·1	3·6	20	31034
tu–A_2 (ricomb.)	0·0	41·0	44·3	12·8	1·9	20	32963
tu–B_3 (ricmob.)	0·0	29·9	46·2	21·0	2·9	20	15992
ltr/ltr	–(*)	48·2	37·8	12·0	2·0	30	6232
ltr/Sb Me′	0·0	19·7	71·0	6·2	3·1	24	37530

The case of tu B_3 deserves a special consideration. In this stock the lymph gland releases some isolated cells during the whole larval stage, but, beside this, entire lobes detach and melanization occurs only inside them. In other words, large cells melanize without being released from the gland, This variant is not the only known. In tu-mwh (Castiglioni, personal communication) the lymph gland is severely dissolved, and many cells released, but the melanization occurs inside the gland lobes which remain attached to the gland. In this stock also pretumours are made up of elongated cells ; simultaneously, in some individuals the imaginal discs become irregular, development stops and death follows. The connection between lethality and production of tumours remains entirely obscure.

TABLE 3

Percentages of the large cells related to the genotype and to the body length of the larvae

Genotype	length of the larvae in mm. 2–2,5	2,5–3	3–3,5	3,5–4	4–4,5	4,5–5	5–5,5	5,5–6	6–6,5
Varese	–	–	2·4	3·9	2·8	1·4	–	–	–
S. Maria	–	–	1·2	3·3	1·2	2·6	–	–	–
b cn vg	–	–	2·5	6·1	5·1	2·0	–	–	
tu–A_2 (2° cr. tu)	–	–	33·9	13·0	10·9	6·0	–	–	–
tu–B_3 (2° cr. tu)	–	–	9·7	12·9	12·1	23·2	–	–	–
mwh–tu	–	–	43·6	12·4	14·2	16·1	–	–	–
SoC–tu	10·6	12·0	7·2	2·6	4·5	–	–	–	–
ltr/ltr	–	–	–	–	18·8	11·4	11·2	14·8	9·3
ltr/Sb Me′	–	–	8·8	6·8	6·6	5·3	–	–	–

The list of different pathways through which the lymph gland and the haemolymph cells pass to reach the melanization phase shows clearly a considerable variety. Although the present paper is mainly concerned with the reference of data of our group, a mention must be made of the case described by El Shatoury (1955), where malignant growth is claimed to occur, and that of tu^w (Rizki, 1961), where the melanotic masses are produced in the caudal fatbody, from aggregations of lamellocytes (according to Rizki's nomenclature, possibly corresponding to the large cells of Castiglioni). In the stock Freckled a similar phenomenon may be also present.

The relationship between larval age and melanization :

The relationship between larval development (expressed as larval length) and cell number (expressed as total number and relative frequency of the different cell types) has been already analysed. We must now consider the relationship between larval development and melanization. A first datum is gathered classifying larvae in tumours and non tumours at different length. One remarks (Table 4) that the genotype controls very clearly the phenomenon. The actual growth of a tumour starts when the melanine is sufficiently abundant as to be seen at a magnification of 40 times. Inspecting the table, one sees how late is tumour growth in tu A_2 and, conversely, how early, tu–So^C and in tu–w. No connection seems to exist between the length at which the first tumour appears and the frequency of tumour present at the end of the larval stage. One example is known (Freckled) where the adults show 100% of tumours and no tumours are detectable before pupation.

TABLE 4

No. of larvae having at least one pseudotumour belonging to different stocks

length : mm.	0·5–1 0	1·0–1·5	1·5–2·0	2,0–2·5	5·2–3·0	3·0–3,5	3·5–4·0	4·0–4·5
Stocks : tu A_2	0	0	0	0	2	15	53	89
,, : tu B_3	0	0	0	2	15	37	58	87
,, : tu C_4	0	0	0	0	15	24	55	83
,, : tu D	0	0	0	0	8	25	52	60
,, : tu–w	7	8	9	9	9	18	14	15
,, : tu–y Hw	2	3	5	4	8	10	8	13
,, : tu–NB^{-S}	6	6	7	5	7	12	23	18
,, : tu–mwh	1	3	3	6	9	32	38	38
,, : tu–So^C	8	17	20	33	22	9	16	5

A special consideration deserves stock tu–So^C, where tumour incidence decreases in the largest larvae, if compared with the incidence in earlier stages. The reason of this fact is not clear : possibly a proportion of tumorous larvae dies at a length of 2·5–3·0 mm.

The number of melanotic masses is difficult to assess precisely, even in dissected larvae, because, in several cases, it is difficult to distinguish between one single large and irregular mass and many small masses tied together ; nonetheless some attempts have been made. Comparing different larval lengths one finds a different incidence of larvae having more than 4 tumours.

From Table 5 we reach two conclusions: 1st, that between stocks there is a strong difference as far as the existence of multiple melanizing points is concerned, 2nd, that a large part of the genome seems to be involved in the process, if there is such a big difference between the original stocks and the genotypes derived from them, where only the 2nd original chromosome is present.

TABLE 5

Percentage of more than 4 tumours per larva

Stocks	length mm 2·0–2,5	4,5–5·0
tu A_2 (original Stock)	–	–
tu A_2 (2nd chrom.)	–	7%
tu B_3 (original Stock)	–	28%
tu B_3 (2nd chrom.)	–	1%
mel e 144	–	–

To the data shown on the table one may be added, there is further incrase in tumorous individuals during pupation, also in stocks having an early start in the phenomenon. The stock where melanization is continued most strongly after the larval stage is melanotic e 144; tu B_3 (original stock) shows also an increase, but in the devived stock $\frac{+}{+}\ \frac{tu\ B_3}{tu\ B_3}\ \frac{H}{Sb\ Mc}$ melanization stops after the end of the larval stage. In tu A_2 the situation is entirely different: in *both* cases (original and derived stock) there is an increase in tumour incidence between old larvae and adults. In Freckled melanization is restricted to pupal stage.

Environmental influences on tumour manifestation:

Different external conditions exert an influence on tumour development.

Nourishment conditions are important in some stocks, and of no relevance in others. Tu A_2, tu B_3 and several other stocks give the same frequency of tumours fed on the usual cornmeal—glucose—agar—yeast composition, while in one stock (Glass and Plaine, 1955) supplementation with tryptophane increases the tumour percentage very strongly (from 4·4% to 6·2%). Similar results have been obtained by Hartung (1955), who demonstrated a strong activity of indoleacetic acid not only in a tumorous stock (from 1–3% to 11·15%), but also in two nearly tumourless stocks, where a concentration of 3 gr/100 cc. of indoleacetic acid added to the food gave an incidence of 28·19% and of 51·92% respectively. Obviously these melanotic formations were purely somatic and not transmitted. Substances of unknown nature produced from different species and varieties of yeasts are also capable of increasing the tumour incidence (Mitther, 1951). More recently Kanehisa (1956, 1957, 1960 *a b*) brought new informations on the same phenomena involving tryptophane metabolism, the importance of metals and indole acetic acid.

Another condition proved to stimulate tumour production, *i.e.* growing several generations of flies on the same food amount. This causes a higher tumour incidence in the last generation, if compared with the first one. This has been found in three stocks for the first time by Barigozzi (1954), proving that in the old food as a result of chemical and physical changes, or of an "infection" brought

about by some factor released by the flies and accumulating in the food, there i: something favourable for producing melanotic tumours.

Freckled is also strongly sensitive to temperature and, to a lesser extent, to food. Reared at 20° the manifestation is very weak, while at 25° it raises nearly 100%. Supplementation with fresh yeast increases also the penetrance.

In short, the inherited melanotic tumours are greatly influenced by the environment.

Discussion :

The general conclusion we may draw is that the production of melanotic tumours, the last event in a series of facts beginning with differentiation of the haemolymph cells, is more characterized by production of intercellular substance than by cell multiplication. This peculiar form of growth (expressed in term of increase in number of melanotic masses more than in term of enlargement of the individual tumours) is strongly controlled by the genotype. This in some cases, is clearly multichromosomal, and thus multifactorial. This is proved by the selection experiments with melanotic e 144 (Halfer 1961), and by the comparison between two different genotypes of tu A_2.

The present data can be discussed in connection with two points. The first deals with the phenomenon itself of the melanotic tumours (pseudotumours), the pecond with the general significance of our findings, in comparison with other rshenomena.

The description of the procedures through which melanotic masses are produced proves that, in spite of the fact that the adult phenotype is often the same, we distinguish several variants. Each variant corresponds to a different genotype ; beside this the same phenotype can be produced by different genotypes. The number of these is thus very high, also if we disregard the influence of the cytoplasm in their transmission (Barigozzi 1962, Barigozzi Halfer and Sgorbati, 1962).

We can now compare the melanotic tumours of Drosophila with other forms of growth.

The known mechanisms of growth are three : cell multiplication, cell size increase, and production of intercellular substances.

The first case is typical of both the embryonic and the neoplastic growth. The second one case be exemplified by the foetal and the postnatal growth of the nervous cell. The third case, finally, is shown by the connective tissue, as occurs in tendons, or by abnormal formations, as in fibromas. It is obvious that the melanotic tumours of Drosophila considered in the present paper belong to the third type of growth. The difference is only due to the substances produced by cell metabolism and to the simpler stucture of the melanine mass, as compared with the collagen fibres. On the other hand, the large haemolymph cells, before producing melanine, show a peculiar behaviour (congregation and changes in form and function) a parallel of which can be hardly found outside the Insects.

Summary :

The author describes the part taken by the haemolymph cells in producing melanotic tumours in several stocks of Drosophila, showing that the development of the melanotic masses is mainly due to assembling of cells and to production of melanine by them, than to cell multiplication.

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STUDIES ON THE EFFECTS OF FORMAMIDE ON AMPHIBIAN EGGS AND EMBRYOS

By

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Introduction :

The fundamental work of Mazia (1955) has elucidated the chemical nature of the mitotic apparatus (spindle and asters) : the fibres are made of globular proteins which undergo linear polymerization by the formation of—S—S—linkages ; the different spindle fibres are held together by bonds split by strong urea, presumably hydrogen bonds.

The importance of the—S—S—bonds for the integrity of the spindle and the asters has been demonstrated by treating the isolated mitotic apparatus with reducing (β–mercaptoethanol) and oxidizing (dithiodiglycol) agents (Mazia, 1955, 1958 *a*, *b*). The effects of the same substances on morphogenesis, in a great variety of biological systems (amphibian and avian embryos, regenerating tadpoles, hydra and planarians, nucleate and anucleate fragments of the unicellular alga *Acetabularia*), have been extensively studied in this laboratory (Brachet, 1959): it was found that β–mercaptoethanol inhibits morphogenesis in all of the systems tested. This fact demonstrates the importance of the—SH$\rightleftharpoons$—S—S—equilibrium for morphogenesis. We have also studied the effects of mercaptoethanol and dithiodyglycol on mitosis during cleavage in amphibian eggs (Limbosch-Rolin and Brachet, 1961) and obtained results similar to those reported by Mazia (1958 *a*, *b*) on the mitotic apparatus isolated from sea urchin eggs : mercaptoethanol dissolves the mitotic apparatus, while dithiodiglycol makes it stiffer, even in living morulae.

During a recent visit to our laboratory, Prof. D. Mazia mentioned the fact that, according to work currently done in his laboratory, *formamide* is superior to urea as a hydrogen bond breaking agent, and that sea urchin eggs withstand high concentrations (up to 2 M) of this substance. It was agreed, at that time, that a study of the effects of formamide on amphibian eggs development would be undertaken in Brussels. The present communication describes the first results obtained in this study.

Material and Methods :

The experiments have been performed on eggs of two different species : *Pleurodeles Waltlii* and *Rana esculenta*. The concentrations in formamide varied between 1 M and 0·01 M, the medium used being that of Niu and Twitty. Eggs were placed at various stages (usually, young morulae or early gastrulae) in formamide and fixed in Zenker acetic at various time intervals. Alternate sections were stained with Unna (methyl green—pyronine) and with the Feulgen reaction.

Only the *Pleurodeles* series has so far been studied cytologically : conclusions must therefore, for the time being, be restricted to this single species.

Experimetal results :

1. *Observations on the living.* The results obtained were essentially the same in 3 experiments on *Pleurodeles* and 2 experiments on *Rana esculenta*. One single description of the results obtained in these 5 experiments will thus be sufficient :

(*a*) *1 M formamide* immediately stops cleavage and gastrulation, without killing the cells before 1 or 2 days. Report in normal medium of embryos treated with 1 M formamide for 15–20 hours doesn't allow further development : under these conditions, the inhibition has become irreversible. Very conspicuous is the fact that, in both morulae and gastrulae, the cells are completely dissociated from each other : concentrated formamide obviously dissolves the coat which links together the cells. The importance of this coat for morphogenesis is now well established (Holtfreter, 1943 ; Curtis, 1960).

(*b*) 0·3 *M formamide* allows cleavage or gastrulation to proceed for a few hours : morulae are blocked as late blastulae ; blastulae form a dorsal lip, which quickly regresses. The effect of 0·3 M formamide becomes irreversible after a 24 hours treatment.

(*c*) *0·15 M and 0·1 M formamide* solutions have the same effects. Development is first normal during a couple of days. At that time, the treated embryos begin to lag behind the controls. After 5–6 days, the embryos are still alive, but abnormal in several respects : the gills and the tail are short ; although the heart is beating, no blood circulation is visible in the gills. The size of the eyes is reduced and the pigmentation of the eyes and body is reduced and patchy. Motility is greatly reduced. After 10–12 days of continuous treatment in 0·1–0·15 M formamide, cytolysis of the gills and the tail begin to show up.

A few *Pleurodeles* gastrulae were cultivated for 8 days in 0·1 M formamide, then transferred back to normal medium for two days : they developed oedema, although their heart was beating more strongly than in the embryos which had been kept continuously (for 10 days) in 0·1 M formamide.

(*d*) *In* 0·01 *M formamide*, development was always normal.

2. *Cytological and cytochemical observations* (*Pleurodeles*)

(*a*) *1 M formamide.* At this concentration, the effect of formamide on *morulae* is dramatic : the mitotic apparatus completely loses, within a few hours, its integrity. Astral and spindle fibres vanish and are replaced by a finely granular material, which retains its strong affinity for pyronine. The chromatin is completely pycnotic and may be eliminated in the cytoplasm ; it still stains strongly with Feulgen and deep blue with Unna (fig. 1). It is clear that, as expected from Mazia's (1955) results, rupture of hydrogen bonds by concentrated formamide leads, even *in vivo*, to the complete loss of the structural integrity of the spindle and asters.

When *yonng gastrulae* are treated with 1 M formamide for 1 or 2 days, the dissociation of the cells, due to the break-down of the coat, is striking. The nuclei are condensed and the nucleoli can no longer be seen after Unna staining. The chromatin stains normally with Feulgen :

but methyl green—pyronine staining clearly shows the existence of a gradient in the penetration of formamide : the nuclei still take up normally the stains in the entoblast (green chromatin), but they stain violet in the outer layers of cells. This violet staining is probably the sign of a denaturation of the DNA molecules, as a result of hydrogen bonds rupture, since Thomas and Durand (1953) have shown that denatured DNA no longer combines with methyl green, but has great affinity for pyronine.

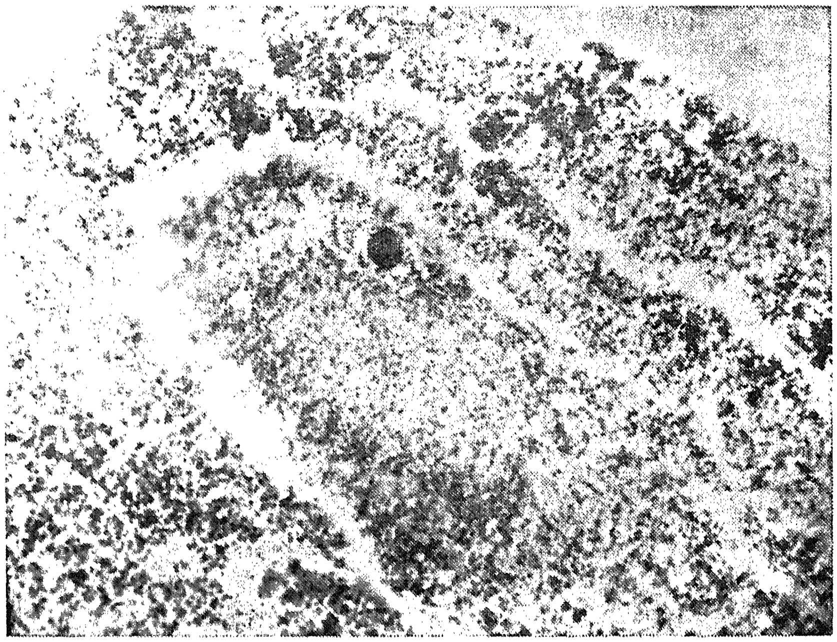

Figure 1. Morula of *Pleurodeles* treated during 12 hours with 1 M formamide ; complete degeneration of the mitotic apparatus and of the chromosomes (pycnosis) Unna staining.

(*b*) *0·1 M formamide.* Gastrulae which have been cultivated in the presence of 0·1 M formamide during 1 or 2 days develop into normal neurulae ; however, a reduction of the size of the nucleoli and the presence of pyronine—staining (reddish) nuclei in the dorsal ectoderm, above the spinal cord, are noticeable.

Much more important alterations are observed when gastrulae are treated for 6–10 days with 0·1 M formamide : there is a strong overall decrease in pyronine staining, both in the nucleoli and in the cytoplasm, suggesting an inhibition of RNA synthesis. Many nuclei become pycnotic, especially in the nervous system, the eyes and the lenses : this wave of pycnoses explains the reduction in size of the eyes and lenses which could be observed *in vivo.* Simultaneously mitotic activity comes to a standstill. Especially striking are the cells of the gill epidermis : their cytoplasm becomes filled with

Feulgen positive granules, which stain red with methyl green pyronine (fig. 2). The possible meaning of these DNA containing cytoplasmic spherules will be discussed later. A long treatment with 0·1 M formamide also results in a considerable inhibition of yolk utilization : the muscles, for instance, are filled with yolk platelets and lack myofibrils, a fact which explains the very poor contractility of the treated tadpoles (fig. 3). The endoderm doesn't differentiate and contains large aggregates of yolk platelets which have fused together. This inhibition of yolk utilization is probably responsible for the complete absence of red blood cells : in fact, no blood forming cells differentiate in the ventral mesoderm, which remains filled with yolk platelets. Finally, it should be mentioned that the chorda has always been found normal : we had expected to find abnormalities similar to those described by Fautrez (1951), who studied the effects of urea on frog eggs and found a "chordalization" of the neighbouring organs (nervous system, somites, dorsal part of the endoderm). Future work will show whether this difference between the results of Fautrez (1951) and ours is due to the use of different amphibian species as a test material, or to different actions of urea and formamide.

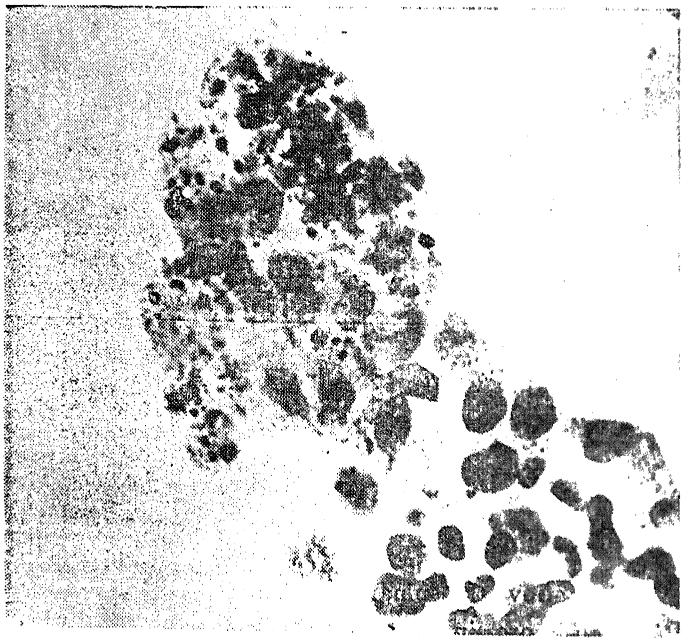

Figure 2. Young gastrula of *Pleurodeles* treated during 8 days with 0·1 formamide. Presence of numerous Feulgen positive granules in the cytoplasm of the cells in the much reduced grills.

Finally, gastrulae which have been treated during 8 days in 0·1 M formamide and then replaced for two days in normal medium definitely differ from those which have remained for 10 days in 0·1 M formamide : despite a large oedema, they look more normal :

there are few pycnoses, the resorption of the yolk has made considerable progress and the differentiation of the muscles and the gut is much better. A few red blood cells can be found in the circulatory apparatus. Therefore, even after a 8 day treatment with 0·1 M formamide, marked reversibility can be obtained after transfer in normal medium.

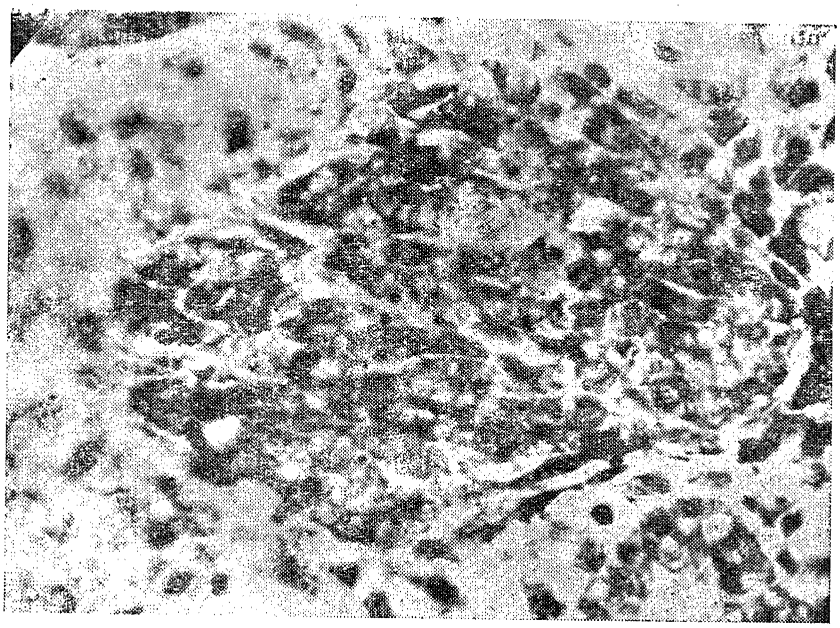

Figure 3. Same as for figure 2, but Unna staining : poor differentiation of the muscle cells, which remain filled with yolk platelets.

Conclusions and Summary :

1. Amphibian eggs withstand high concentrations of formamide, a hydrogen bond-breaking agent. In this respect, formamide and the thiol reagents previously studied in our laboratory (mercaptoethanol, dithiodiglycol) differ considerably. For instance, the impressive effects exercised by mercaptoethanol on neural plate closure (Brachet, 1959) are never obtained with formamide. Reduction of—S—S— group by mercaptoethanol and rupture of hydrogen bonds by formamide have entirely different effects, so far as morphogenesis is concerned.

2. Concentrated (1 M) formamide stops mitosis in cleaving eggs, by bringing out a complete destruction of the astral and spindle fibres. At gastrulation, it also inhibits mitosis and modifies the staining properties of DNA with methyl green-pyronine : the nuclei of the treated embryos stain red or violet, a fact which suggests that DNA has undergone a certain amount of denaturation. Formamide also exerts a powerful effect on the coat which links the cells together : this coat undergoes disruption and the cells dissociate from each other.

3. At lower concentrations (0·1 M), formamide inhibits both DNA (arrest of the mitotic activity) and RNA (reduced nucleolar and cytoplasmic basophilia) synthesis. It also strongly inhibits yolk utilization, especially in the muscles and

the gut. Of special interest perhaps is the occurrence, in the ectodermal cells of embryos which have been treated for 8–10 days with 0·1 M formamide, of cytoplasmic Feulgen positive inclusions. These inclusions recall the DNA—containing granules observed by Chèvrement (1960) in the cytoplasm of tissue culture cells treated with acid deoxyribonuclease. Further experiments, especially autoradiography studies of the incorporation of labeled thymidine, are required before we can safely conclude that we are dealing with a case of cytoplasmic DNA synthesis. If so (and this is a very likely interpretation of the present results), a satisfactory explanation of Chèvremont's (1960) puzzling observations could be given : in cells treated with agents which produce a partial denaturation of DNA (such as deoxyribonuclease, formamide, etc..), single-stranded DNA might be eliminated in the cytoplasm and serve as a primer for DNA polymerase. The result of such a process would be the synthesis of cytoplasmic DNA. In fact, this hypothesis has already found support in recent experiments by Prescott *et al.* (1962), who found that the uptake of primer DNA by pinocytosis is followed with cytoplasmic DNA synthesis in amoebae. Experiments designed to test the hypothesis are in progress in our laboratory.

Acknowledgments :

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ASPECTS OF THE DEVELOPMENTAL GENETICS OF THE LEGS OF DROSOPHILA[1]

By

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The different pairs of legs of the fly *Drosophila melanogaster* are characterized by specific patterns of bristles. Different genotypes are known which affect these patterns in various ways. The analysis of the effects of these genotypes offers a means of studying alternative modes of differentiation.

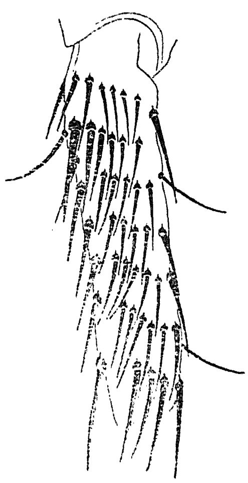

Figure 1*a*. Normal female (from Tokunaga, unpublished).

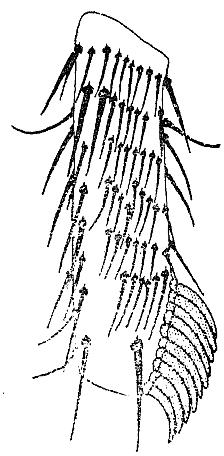

Figure 1*b*. Normal male (with sex comb ; from Tokunaga 1961).

Ventral view of the basitarsal segment of normal male and female in *Drosophila melanogaster.*

One natural alternative of such differentation is the difference between the bristle patterns on the first tarsal segment, the basitarsus, of the foreleg in the two sexes. In females the ventral side of this segment presents approximately eight transverse rows of bristles (macrochaetae) which point toward the distal regions of the leg (Figure 1*a*). In males, only approximately six transverse rows are found but a special structure, the sex comb, is present which is absent on the female basitarsus (Figure 1*b*). The sex comb is a longitudinal row of unusally heavy bristles, arranged like the teeth in a comb. They point more or less across the width of the basitarsus, *i.e.* in a direction quite different from the macrochaetae.

[1]Supported by grant 0-15460 from the National Science Foundation.

The question arises : how do the two genotypes, $1X$=male vs. $2X$=female, control these two different types of differentiation ? A complete answer to this question would, of course, involve a full knowledge of all biochemical properties in which male and female cells differ and an analysis of those differences which are relevant to the different patterns of the basitarsi. Such an analysis would take account of the fact that male constitution of cells by itself does not lead to the differentiation of teeth and sex combs. After all, most cells of a male do not form sex comb teeth, even if they are part of the epidermis. Only those few cells present in a very limited area of the first basitarsus actually do form the sex comb. The original problem can thus be expressed in a different way. In what fashion does the region which differentiates the sex comb in the male vary from the same region which in the females fails to form this organ ?

Two main possibilities were envisaged which would form the basis of an answer to this question. On the one hand, it was possible that a male and a female genotype result in such differences in the organization of the developing imaginal disk of the foreleg that the special properties which must precede the later differentiation, in a specific region, of the sex comb are existent in the male only. On the other hand, it was possible that male and female imaginal disks alike possess a unique region related to sex comb formation but that only male cells are able to respond to the stimulus represented by the region. These two possibilities are familiar from experimental embryology. The amphibian organizer, for instance, represents a specific, unique region in the early embryo, inducing among other differentiations the formation of the neural tissue This induction, however, is dependent on the presence of cells competent to react to the stimulus of the organizer. In these terms, the two possibilities defined in regard to the sex comb can once more be restated as follows : do the specific regions on male and female basitarsi differ in having or not having an inducing property, or are they alike in this property but unlike in their competence to react to it ?

In *Drosophila* the experimental approach to this problem consisted in the study of basitarsi in which cells of male and female genotypes coexist in gynandromorphic patterns. Such sex mosaics can be obtained from eggs which after fertilization possess two X chromosomes but in which one of these, of ring shape, has a tendency to get lost in the cytoplasm during mitotic divisions. The resulting embryo has both 2X and 1X cells, mixed in variable fashion from one individual to another as made apparent by the use of sex-linked genes serving as markers. Some of the gynandric basitarsi were predominantly male with a small area of female tissue occupying part of the region of the sex comb (Figure 2). Others were predominantly female with a small area of male tissue covering part of the corresponding region (Figure 3). If the male disk, in contrast to the female, were unique in providing the sex comb forming stimulus the predominantly male basitarsi should have formed sex comb teeth from either 1X or 2X cells ; and if the female disk did not provide the stimulus neither 2X nor 1X cells should have formed teeth. In contrast, if all basitarsal disks would furnish the stimulus to tooth formation but with competence to respond restricted to 1X cells, then predominantly male tarsi should fail to form teeth where the 2X tissue intruded into the potential sex comb region ; and predominantly female tarsi should form teeth, or a tooth, where 1X tissue was present in that region. The experiment gave a clear decision. Male and female basitarsal disks both provide the stimulus but only male tissue can respond to it (Stern and Hannah 1950).

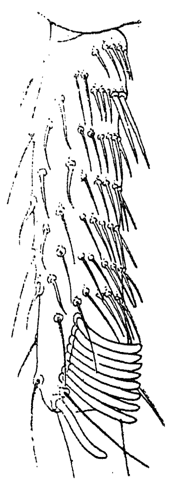

Figure 2. Mosaic basitarsal segment with predominantly male tissue. Female tissue resulting in female bristle within the gap between 1 tooth and 8 teeth respectively (from Stern and Hannah 1950).

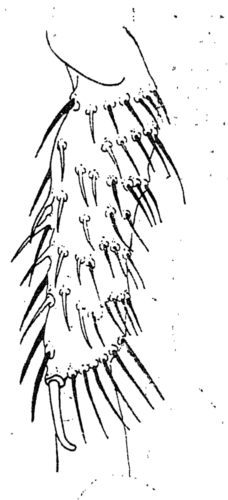

Figure 3. Mosaic basitarsal segment with predominantly female tissue. Sex comb consisting of one tooth (in abnormal position; from Stern and Hannah 1950).

The nature of the stimulus is unknown. It should perhaps not be called an "organizer for sex comb differentiation". Such a designation might imply that there is a direct relation between the properties of the special area and the formation of the comb. To be neutral, we prefer the term "prepattern" which implies no more than some kind of differentness which singles out one region of the leg from others, perhaps a rather trivial biochemical difference or a physical one involving a fold in the imaginal disk or some other singularity.

The ever-present genotypic difference of the two sexes is only one of the tools for this study of developmental genetics. Mutant genes are known which offer other approaches. One of these is the recessive gene engrailed (*en*) which among other effects causes the appearance of a secondary sex comb on the first basitarsus, located like a mirror image in relation to the primary comb (Figure 4). Once again the question is raised whether the presence of homozygous *en/en* changes the configuration of the leg disk in such a fashion as to create a new prepattern at the site of the secondary sex comb or whether all basitarsi possess this prepattern whose presence is only revealed when *en/en* endows the cells with the competence to respond to it. Using a technique for inducing somatic crossing over it is possible to produce homozygous *en/en* spots on heterozygous *en/+* males (Figure 5). These spots, when located in the area of the potential secondary comb, form teeth as if the whole fly were an *en/en* male (Tokunaga 1961). Clearly, unrecognized until the finding of the mutant *en*, every male has a pre-pattern for the secondary sex comb but only *en* cells are competent to respond to its existence. Moreover, the same prepattern exists in female forelegs as shown by the appearance of secondary teeth produced by small male patches in gynanders of *en/en* genotype (Stern 1954).

At this point, a discovery must be reported which throws light on some properties of the prepattern for the primary sex comb (Tokunaga 1962). Studies

on the cell lineage of mosaic basitarsi have shown that the sex comb arises as a distal transverse row which in the course of development shifts toward a longitudinal position to occupy the position of the differentiated comb. This shift makes understandable the direction of the teeth. Apparently their direction as vertical to the initial transverse row is determined before the rotation of this row so that after rotation the direction of the teeth themselves differs greatly from that of the macrochaetae of the normal transverse rows.

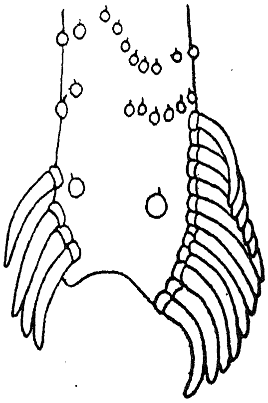

Figure 4. Distal part of basitarsal segment of homozygous *en* male foreleg (from Tokunaga 1961).

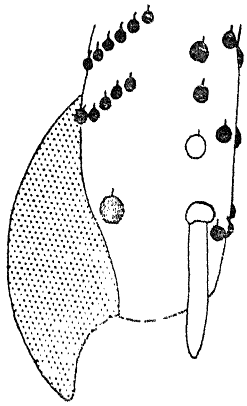

Figure 5. Mosaic distal part of basitarsal segment with *en/en* spot in *en/+* leg (from Tokunaga 1961). Stippled area represents the primary sex comb, the single tooth on the right is a secondary sex comb tooth.

Gynanders in which only a very small part of the relevant basitarsal region is male show that differentiation of teeth and rotation are independent processes. Small male areas do not rotate yet differentiate typical teeth. The same conclusion can be drawn from the study of the effect of the sex-linked mutant gene sexcombless (*sx*). As the name indicates, *sx* males do not have a sex comb. Nevertheless, some of them have one to several teeth, more or less scattered over the distal region of the basitarsus and often directed parallel to the macrochaetae or at only a slight angle to them. Sexcombless then seems to reduce the competence of cells to respond to the prepattern. As a result, no rotation of a transverse row takes place although some shifting of direction of limited cell areas may occur (Figure 6). The reduction of competence is also borne out by the fact that the teeth in sexcombless may differentiate in various degrees, from typical teeth to structures intermediate, in shape and pigmentation, between teeth and ordinary macrochaetae. This finding is in contrast to that concerning the sex combs in triploid intersexes. These have fewer teeth than normal males but each tooth is fully formed (Hannah and Stern 1957).

At first sight, the gene eyeless-dominant (*ey*D) seems to have the opposite effect from that of *sx*. It greatly increases the number of sex comb teeth. A morphologic study, however, suggests a different interpretation. The sex combs of *ey*D males are "about doubled in size" (Patterson and Muller 1930) not by having a larger row of teeth or by irregular scattering of numerous teeth over the area of the sex comb but by forming two, three or more sex combs oriented more or less parallel to one another (Figure 7). These combs are usually crowded upon one another so that they are not always clearly defined as separate elements.

Their origin seems to be correlated with the fact that the second tarsal segments of ey^D flies are "shortened to give lumps" (Bridges and Brehme 1944). This abnormal morphology is found in both sexes and is caused by a frequent inability of the leg disk to form separate proximal tarsal segments. In many ey^D flies the first and second segments are a more or less continuous structure with several incomplete sockets, only one of which is present normally and, if fully formed articulates the basitarsus with the next segment. The lack of separation of the two segments results, in females of ey^D, in the formation of one or more additional transverse rows as compared to normal females. In the male these extra rows are all rotated and thus form multiple sex combs. This then seems an example where a mutant genotype, ey^D, quantitatively changes, by way of incomplete tarsal segmentation, the prepattern of the sex comb area. It enlarges the prepattern so as to involve several transverse rows all of which, if male, respond by rotation and tooth formation.

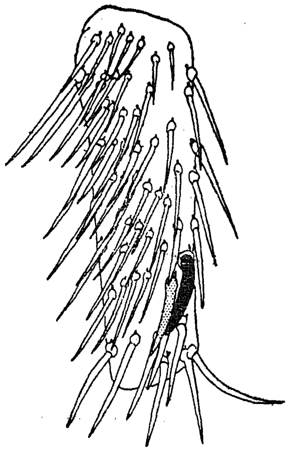

Figure 6. Basitarsal segment of *sx* male foreleg with one typical tooth. Note the dark intermediate bristle-like tooth adjacent to the typical tooth (stippled).

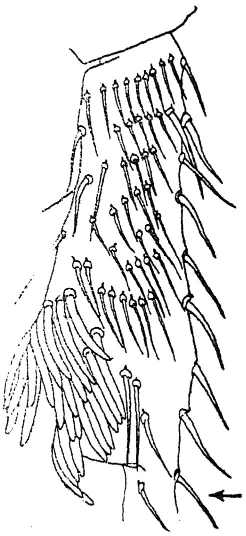

Figure 7. Basitarsal segment of ey^D male foreleg. Arrow indicates proximal part of the second tarsus incompletely separated from the basitarsus.

Our experiments include the production of various combined genotypes, such as sexcombless eyeless-dominant, sexcombless engrailed and others. In the former combinations *sx* is epistatic to ey^D as far as the formation of teeth is concerned, there being on the average only one more tooth than in *sx*. The shape and faulty segmentation of the legs is not affected by *sx*. This is in agreement with the hypothesis that *sx* reduces the competence of cells to respond to the prepattern.

The combination *sx en* shows an unexpected interaction effect. Usually the whole tarsus consists of only a single or of two sections as if all or most tarsal segments had been fused. The number of transverse rows in the proximal region, corresponding to the basitarsus, is within the normal range but their width is greatly increased. Indeed, most rows are made up of two parts separated by a gap, one part being like that typically located between the longitudinal rows

f and 7, and the other consisting of a new series of transverse rows lying intercalated between longitudinal row 1 and the typical transverse rows (Figure 8). These facts fall in line with the appearance of the secondary sex comb with its mirror image location in respect with the primary comb. In *sx en* flies there are formed not only secondary sex comb teeth but also a whole series of secondary transverse rows. The formation of these secondary rows is correlated with the increased width and a shortening of the tarsus. The *sx en* genotype changes the prepattern for transverse row formation.

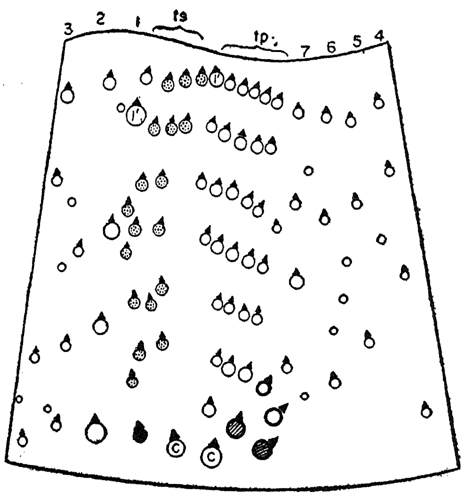

Figure 8. Schematic representation of the bristle pattern on the first tarsus of a *sx* ; *en/en* male foreleg. Heavy circles = primary sex comb teeth ; light circles = normal bristles ; solid circles = secondary sex comb teeth ; dotted circles = secondary series of transverse row bristles ; hatched circle = intermediate dark macrochaeta. The triangular symbols next to most chaetae are the "bracts".
1, 2, 3, 4, 5, 6, 7 = numbers assigned to longitudinal bristle rows ; t_p = primary transverse rows (on the ventral side of the tarsus) ; t_s = secondary transverse rows ; C = central bristles.
The basitarsal cylinder is represented in one plane, as if it had been cut between rows 3 and 4 and laid out flat.

The genotypes hereto discussed affect sex comb differentiation in the first leg. There are others which cause the appearance of sex combs on the second and third legs which normally do not carry sex combs. As shown by Hannah-Alava (1958) genes for such extra sex combs, on other than the first legs, are homoeotic mutants. In varying ways they tend to transform the middle and hind legs into oreleg-like structures (Figures 9 and 10). These findings suggested that extra sex comb genes act by changing the whole pattern of leg development and that the apearance of the extra combs was not due to the invariant existence of comb prepatterns on all legs but due to the creation of such prepatterns in the homoeotic legs. Matters, however, seem to be more complex. Unpublished data of Dr. C. Tokunaga on mosaics demonstrate the differentiation of teeth on second and third legs independently of homoeotic transformation of these appendages. A full understanding of these phenomena will have to await still further study.

This survey of work on a small area of the legs of *Drosophila* has been presented as an illustration of how a combination of genetic and developmental methods may serve to illuminate the problems of genic control of differentiation.

In the most recent terminology originally designed for processes of control of gene action in microorganisms, it may be suggested that in a developing organism regional singularities, prepatterns, arise which serve to control the genes concerned with differentiation. A given gene is inactive or nearly so in many parts of the developing system but is stimulated to activity in special sites. And different alleles of a gene either endow it with the capacity to respond to the call for action or not to respond. Either one of these alternatives determines the fate of the developing part and indirectly affects others.

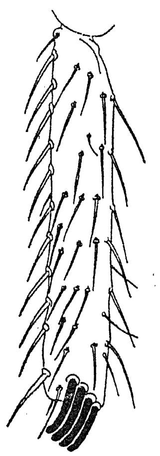

Figure 9. An intermediate case of a transformed basitarsal segment of the second leg with four typical teeth. The newly induced or transformed bristles of the transverse rows are in solid black ; the bristles characteristic of the second leg are in outline (from Hannah-Alava 1958).

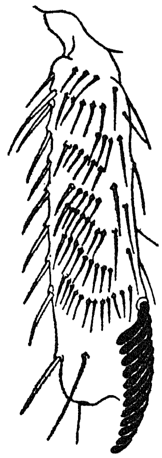

Figure 10. An extreme case of a transformed basitarsal segment of the third leg with twelve teeth. The newly induced or transformed bristles of the transverse rows are in solid black ; the bristles characteristic of the third leg are in outline (from Hannah-Alava 1958).

Summary :

The differentiation of bristles follows specific patterns in different pairs of legs of the fruitfly *Drosophila melanogaster*. The differentiation of the "sex comb" in the forelegs is an example of such specific pattern. The first tarsal segment (basitarsus) in the male differentiates a sex comb in a limited distal area ; in the female it does not develop any sex comb. It has been possible to establish, by means of mosaic spots and gynanders, that the male and female basitarsi both provide the stimulus for the formation of the sex comb but that only the male tissue can respond to this stimulus.

The localized stimulating area is a "prepattern", a term which implies some kind of differentness which singles out one region of the leg from others. By means of various mutant genes different patterns can be studied. Using such mutant genes as engrailed (*en*), sexcombless (*sx*), eyeless-dominant (*ey*D) and extra-sexcomb (*esc*), it has been possible to study the patterns of two different sex combs in the basitarsal segment of *Drosophila melanogaster*, the primary and secondary sex combs respectively. Some mutant genes act at the level of the prepattern, others at the level of the competence of cells to respond to the prepattern. For instance, the gene eyeless-dominant quantitatively changes the prepattern by way

of incomplete segmentation of the tarsus, while sexcombless reduces the competence of cells to respond to the prepatterns for the formation of the primary and secondary sex combs. From all these facts it may be suggested that in a developing organism regional singularities arise which serve to control the genes concerned with differentiation. Activation of the specific gene at a specific place and its inactivation at many others is dependent upon such regional singularities, the prepatterns.

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REGIONAL ORGANIZATION IN THE AMPHIBIAN LIMB REGENERATE—A NEW HYPOTHESIS

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Abstract :

Experiments are described involving carbon marking and transplantation to a neutral (non-limb-forming) site, of Axolotl fore limb regeneration blastemas and regenerates, or parts of regenerates. On the basis of the results a hypothesis is presented to explain the origin of the proximo-distal pattern of organization in the regenerating limb. According to this hypothesis the dedifferentiated cells of the early blastema possess, as an intrinsic property, tendencies for differentiation into *distal* limb structures. The distal part of the regenerate (roughly the hand and wrist) would develop autonomously according to these intrinsic tendencies, whereas the lower and upper arm regions would arise under the influence of the stump. The importance is stressed of the intrinsic morphogenetic capacities of morphologically undifferentiated cells as primary factors in the establishment of structural patterns during morphogenesis.

If an amphibian limb is amputated through the region of the stylopodium, a blastema forms on the amputation surface, by accumulation under the wound epidermis of mesenchymal cells. These cells originate from tissues of the limb stump through a process of dedifferentiation. Soon the blastema starts growing, and after a while the three main limb regions, stylopodium (upper limb), zeugopodium (lower limb), and autopodium (hand or foot), differentiate in a roughly proximo-distal order. The fact that the proximal structures differentiate first has led older authors to believe that the establishment of the typical proximo-distal limb pattern in the regenerate would be due to a chain of interactions in which each region would determine the differentiation of the region distal to it. This chain would start from the differentiated tissues of the stump and proceed distalwards. On the basis of their experiments, other authors (*e.g.* Weiss) developed the idea that the differentiation of the regenerate is governed by a morphogenetic field. This field was envisaged as being an extension of the "limb field" present in the stump. This limb field in its turn would be a remnant of the embryonic "individuation field" of the limb. Common to both these concepts is the idea that the blastema is at first a passive, morphogenetically neutral mass of cells, upon which a pattern is gradually imposed by the stump.

Mettetal (1939), in an unfortunately rather neglected publication, was the first to direct attention towards the active role played by the blastema in the establishment of the limb pattern. He found that early limb blastemas, when cut from the stump and transplanted to an environment neutral with regard to limb-forming potencies, surprisingly gave rise to digits only, not, as one might have expected, to proximal limb structures.

For a re-evaluation of Mettetal's results it was necessary to find out first which is the later fate of the mesenchyme of such early blastemas.* To this end

*In this paper we will only speak about the mesenchyme of the blastema, and disregard the epidermis, whose role in the process of proximo-distal regional organisation is probably largely unspecific.

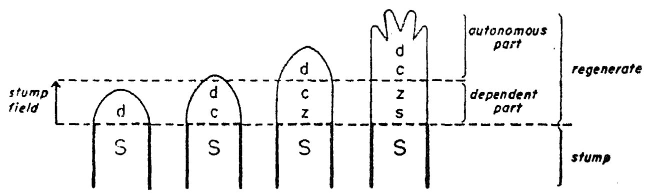
a mark of carbon particles was inserted with a fine steel needle into the mesenchyme at the tip of an early conical blastema of the Axolotl fore limb. During the early growth of the regenerate the mark does not stay at the tip, but new tissue appears distal to it. In the differentiated regenerate the mark is always found to lie somewhere in the zeugopodium, usually near the elbow. It follows that the blastema grows mainly by proliferation at the tip. The material for the entire autopodium and part of the zeugopodium is laid down by an *apical proliferation centre.* The prospective fate of the bulk of the mesenchyme at the early cone stage is to form the most proximal limb parts. It is the more surprising that upon transplantation this material should form digits only, as Mettetal found.

Still, this finding could be corroborated in our experiments. Blastemas of different stages were auto-transplanted to the back, *i.e.* to a region where no limb-forming potencies are normally found. Four stages were used : (I) a small conical blastema, (II) a larger conical blastema, (III) an early paddle-shaped blastema, and (IV) a late paddle stage with an indication of the first digit. In stage IV the rudiments of the humerus, the radius and ulna, and the first digit are already laid down. Stage I yields only one or two digits ; in transplants of stage II carpal elements appear in addition to the digits. Stage III gives rise to several cases with a zeugopodium, while stage IV finally sometimes forms a small but qualitatively complete limb. At the same time the average number of digits and of skeletal elements formed rises with advancing stage of transplantation. It is significant that in most transplants, regardless of the stage, more distal structures (digits, phalanges) predominate numerically over more proximal ones. It seems that in general the amount of mesenchyme available is preferentially used for the formation of *distal* structures, and that more proximal ones have a chance of appearing only if the amount of material available is sufficient. This, by the way, does not say anything about the subsequent order of differentiation. The phase of differentiation is preceded by a phase of determination. During the early determination of the limb pattern in the undifferentiated mesenchyme tendencies to form distal structures seem to take the lead (not, as one might perhaps expect, tendencies to form proximal structures).

Our conclusion from these results has been that the mesenchyme of the early cone-shaped blastema possesses *intrinsic* tendencies for differentiation in a *distal* direction, notwithstanding the fact that this material is normally destined to form proximal limb structures. This conclusion is corroborated by the following experiment. A blastema of the late paddle-shaped stage (stage IV) is divided into a distal and a proximal half of about equal volume, and these halves are transplanted side by side. The distal half, as might be expected, yields only digits and carpal elements. The proximal half, which contains the rudiments of the humerus and of the radius and ulna, nevertheless also gives rise exclusively to digits and carpal elements. The only way to account for this surprising result is to assume that under the influence of the distal wound surface the rudiments already laid down in the proximal half dedifferentiate, and the entire transplant reverts to an early blastematous state. In the process of dedifferentiation the cells apparently again acquire tendencies for distal differentiation. The further development is then characterized by the establishment of a new apical proliferation centre, leading to growth of the transplant followed by digit formation.

Our general conclusion thus was that limb cells which undergo a process of dedifferentiation, thereby *automatically* acquire intrinsic tendencies for distal differentiation. It will be clear that this applies also to the cells of the early cone-shaped blastema, which have arisen by dedifferentiation from stump tissues.

The question now remains why the mesenchyme of the early blastema, in spite of its intrinsic tendencies for distal differentiation, normally forms proximal limb structures instead. Now it should be remembered that this material stays in contact with stump tissues during the later phases of normal regeneration. Apparently it is the influence of the stump which gradually changes the distal differentiation tendencies of the material into tendencies for proximal differentiation. At the same time more material with intrinsic distal differentiation tendencies is added at the tip of the blastema by the apical proliferation centre. It is assumed that the stump influence extends into the blastema with decrement. The stump influence would thus constitute a *stump field* with limited extension. Consequently the tip of the growing blastema would gradually "escape" from the influence of the stump. Ultimately the entire distal part of the regenerate (roughly the entire autopodium, *i.e.*, carpus and digits) would develop autonomously. The establishment of its structural pattern would be governed by an *apical organization centre*, which may be conceived as being identical with the apical proliferation centre. The entire concept may be summarized in the following diagram.



S = stylopodium of stump
d, c, z, s = diff. tend. for digits, carpus, zeugopodium, stylopodium

This concept may explain several results previously obtained by other investigators. First of all there are the results obtained by letting regeneration proceed from a longitudinally halved stump (Weiss, 1926), or from a double stump obtained by grafting two limbs together longitudinally (*e.g.* Goss, 1956). In a number of cases such stumps give rise to single, whole autopodia, while the more proximal structures of the regenerate are either defective (on a half stump) or partially double (on a double stump). If, as we suggest, the apical organization centre arises and acts independent of the stump, in either case one such centre would be formed, which would condition the formation of a single, whole autopodium, and there is no reason why a half or a double autopodium should arise. On the other hand, the more proximal limb structures would be under continuous, direct influence of the experimentally altered stump, and consequently would be either defective or double.

In consequence of this view the morphological regulation which is observed at the level of the autopodium would not be the result of "regulative properties" of a hypothetical limb field, but simply the consequence of the morphogenetic independence of the autopodium.

Next we may mention the results obtained with limbs transplanted with the proximo-distal axis reversed (*e.g.* Dent, 1954). If such a limb is subsequently amputated through the stylopodium, the regenerate which arises on the wound surface (which is directed proximally with regard to the original orientation of

the limb) will in general contain all structures which are *distal* (not proximal) with reference to the level of amputation, in other words, stylopodium, zeugopodium, and autopodium, notwithstanding the fact that all these structures are already present in the stump. In the light of our hypothesis this result is not so difficult to understand as it seems at first sight, since the presence of an amputation surface, whatever its orientation, will always lead to the formation of a blastema by accumulation of dedifferentiated cells, which will automatically acquire tendencies for differentiation in a distal direction. The additional information to be derived from this type of experiment is that what we have called the stump field is clearly not an extension of a hypothetical limb field still present in the stump. The character of the stump influence appears to be determined exclusively by the level of amputation, or in other words, by the regional quality of the differentiated tissues present in the amputation plane.

There are indications that the same hypothesis may perhaps be applied to the regenerating urodele *tail*. If, for instance, a tail which lacks the dorsal fin is amputated, it is often seen that the resulting regenerate is proximally without a dorsal fin, but that its most distal tip possesses a short stretch of normally shaped fin, which starts rather abruptly at a certain distance from the level of amputation (cf. Newth, 1958). This may mean that the tip is morphogenetically independent of the stump, and consequently able to "regulate", in contrast to the basal part of the regenerate. This problem is currently under investigation in our Laboratory.

The main theoretical significance of the hypothesis lies in the fact that it once more emphasizes that explanations of pattern formation during morphogenesis will always have to take account of two types of morphogenetic factors : (1) factors *extrinsic* to the undifferentiated cellular material in which the pattern arises (commonly called inductive factors), and (2) the primary, *intrinsic* properties of the material in question, which is not necessarily morphogeneticaly neutral or "nullipotent". Patterns probably never arise as the result of either of these two types of factors alone, but always seem to be the outcome of their combined and complementary action.

An illustration of the primary importance of the intrinsic properties of the blastemal mesenchyme is provided by the experiment reported above, where a transplanted proximal half of a paddle-shaped regenerate reorganized itself autonomously into a distal limb segment, consisting of digits and carpal elements. A still more striking illustration is provided by the following experiment. From a similar paddle-stage regenerate the distal third (the digital plate or hand plate) is cut off, and the proximal part is transplanted upside down into a pocket made in the muscles of the back. The skin of the back is allowed to heal over the exposed proximal cut surface of the transplant. In the following days the skeletal rudiments already present in the regenerate undergo dedifferentiation, and the entire transplant reverts to an early blastematous condition (the epidermis of the transplant degenerates). In this unorganized mass of mesenchymal cells apparently a new apical proliferation centre originates at the originally proximal, now outwardly directed side of the transplant, and this leads to the formation of a small outgrowth consisting of limb mesenchyme covered by body epidermis. Ultimately most of these outgrowths develop into one or two digits, sometimes accompanied by a few carpal elements. Thus, a (defective) autopodium with reversed orientation has arisen out of a small amorphous mass of limb mesenchyme by a completely autonomous process of reorganization. During this process again the tendencies for differentiation in a distal direction take the lead, although the

mesenchyme transplanted, if left *in situ* on the stump, would have formed proximal limb parts.*

These findings surely demonstrate that it is difficult to over-estimate the importance of the inherent capacities of organization and differentiation of morphologically undifferentiated cells. The question whether these cells are truly undifferentiated in a physiological (biochemical) sense is a difficult problem, which will not be discussed further at this place.

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